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ORIGINAL PAPER

Single amino acid substitutions in the coat protein and RNA-dependent RNA polymerase alleviated the virulence of *Cucumber green mottle mosaic virus* and conferred cross protection against severe infection

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Abstract
Cross protection is a promising alternate to control *Cucumber green mottle mosaic virus* (CGMMV) which is of increasing economic importance to cucurbit production worldwide. One major factor confronting the application of cross protection to control CGMMV is the scarcity of available mild mutants. The objective of this paper was to screen attenuated mutants of CGMMV and evaluate their potential in cross protection. An infectious cDNA clone of CGMMV, pCGMMV, was obtained by cloning intron-containing CGMMV genome to modified pCambia0390 vector with the *Cauliflower mosaic virus* 35S promoter. Five pCGMMV-derived mutants were obtained via site-directed mutagenesis and inoculated to *Nicotiana benthamiana* plants for symptom observation. The attenuated CGMMV mutants were evaluated for their efficiency in cross protection. The intron-containing clone pCGMMV induced similar disease symptoms and accumulated similar titres of virus in *N. benthamiana* plants as wild-type CGMMV. Mutations of aspartic acid at position 89 in the coat protein to alanine ($D^{89}A$) or glutamic acid at position 1069 in the ORF1/2 read-through protein, in the RNA-dependent RNA polymerase domain to alanine ($E^{1069}A$) alleviated the symptoms of pCGMMV in *N. benthamiana* plants significantly. In cross protection assay, the two mutants pCGMMV-CP-D89A and pCGMMV-RdRp-E1069A could prevent the superinfection of CGMMV, with protection efficiency of 91.7% and 100%, respectively. The intron-containing clone pCGMMV was stable and highly infectious. The D^{89} in the coat protein and E^{1069} in the RNA-dependent RNA polymerase played an important role in regulating the virulence of CGMMV. Mutants pCGMMV-CP-D89A and pCGMMV-RdRp-E1069A were of great potential in the control of CGMMV via cross protection.

Keywords CGMMV · Cross protection · Mild mutants · RNA-dependent RNA polymerase · Coat protein

Abbreviations

bp	Base pairs
CGMMV	*Cucumber green mottle mosaic virus*
CP	Coat protein
MP	Movement protein
ORF	Opening reading frames
PMMoV	*Pepper mild mottle mosaic virus*
PTA-ELISA	Plate-trapped antigen enzyme-linked immunosorbent assay
qRT-PCR	Quantitative real-time polymerase chain reaction
RdRP	RNA-dependent RNA polymerase
ToMV	*Tomato mosaic virus*
UTR	Untranslated region

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Introduction

Cross protection is a phenomenon that plants pre-inoculated with mild or attenuated viral strains will be protected from severe infection by strains of the same or related viral

strains [1, 2]. It is a promising alternate for plant virus disease control, especially when no resistant cultivar available, and has been used successfully to control a variety of viral diseases [3–8].

Cucumber green mottle mosaic virus (CGMMV) belongs to genus *Tobamovirus* of family *Virgaviridae* [9]. The genome comprises a linear positive single-stranded RNA with four opening reading frames (ORFs) and the 5′- and 3′-untranslated regions (UTRs). ORFs 1 and 2 encode a 129-kDa and a 186-kDa protein, respectively, which are RNA-dependent RNA polymerases (RdRp) associated with viral replication. ORF3 encodes a 29-kDa movement protein (MP), while ORF4 encodes a 17.4-kDa coat protein (CP) [10, 11]. CGMMV can be transmitted via mechanical contact and seed, and mainly infects crops of family *Cucurbitaceae*, including *Cucumis sativus*, *Citrullus lanatus*, *Cucumis melo*, *Lagenaria siceraria*, *Luffa cylindrical* and *Momordica charantia*, and cause severe losses to the cucurbit production in many countries [12–16]. CGMMV was divided into three host-specific types according to the results of single-strand conformation polymorphism analysis with coat protein and partial movement protein genes [16]. The phylogenetic analysis with the complete genomic sequences showed that CGMMV was divided into three groups; however, the groups were geographical origin specific, rather than host origin specific [17]. Since its first incidence in China in 2005, CGMMV has been reported to occur in 23 provinces of mainland China and caused huge losses to the production of watermelon and cucumber [17]. The most effective method to control CGMMV is planting resistant cultivars. However, the widely planted cultivars are susceptible to CGMMV, and no resistant stock was available in the practice. Therefore, cross protection has become a promising alternative for CGMMV control. Treatment of CGMMV VIROG-43 with nitrite mutagenesis yield a stable attenuated strain VIROG-43M, which can protect cucumber plants from severe infection [16]. CGMMV-SH33b, an attenuated strain obtained from ultraviolet light treatment showed remarkable effect in protecting muskmelon plants from outbreaks of severe symptoms, and in eliminating the wild-type CGMMV from the greenhouse [18]. The attenuated CGMMV strains were either naturally occurring isolates or obtained by chemical mutagenesis; the underlying pathogenesis mechanism remains largely unknown. The objective of this paper was to obtain attenuated mutants of CGMMV via site-directed mutagenesis and evaluate their potential in controlling CGMMV via cross protection. We first showed that single amino acid substitution in the coat protein and RNA-dependent RNA polymerase significantly alleviated the virulence of CGMMV, and then confirmed two attenuated mutants possess great potential in the control of CGMMV.

Materials and methods

Construction of cDNA infectious clone of CGMMV

The complete genome of CGMMV isolate JN (GenBank accession no. KR232571) [17] was amplified by two overlapping PCR reactions. We first amplified 35S promoter of *Cauliflower mosaic virus* with restriction site Hind III introduced to the 5′-end of it and then amplified a fragment from the first to the 3721th base pair of CGMMV-JN. The fragment 35S-Rd3721 was generated by an overlapping PCR. The fragment Rd3481-3′-UTR contained the 3′-terminal 3481 bp of CGMMV-JN genome and the 3′-UTR, with restriction site XhoI introduced to the end of 3′-UTR. The fragments 35S-Rd3721 and Rd3481-3′-UTR had an overlapping region of 240 bp. The unique restriction site BamHI was introduced to the nucleotide 3699 of the CGMMV-JN genome. The two fragments were digested by BamHI individually and then ligated to obtain the full-length CGMMV-JN genome, which was inserted into the vector pCambia0390, producing the plasmid pCGMMV. In order to stabilize the clone, a 515-bp watermelon intron (GenBank AB006530.1) was inserted to nucleotide 3821 in RdRp-coding region (Fig. 1a).

To test the infectivity of the newly constructed clone, *Agrobacterium tumefaciens* GV3101 was transformed with pCGMMV and agroinfiltrated into the leaves of 6-week-old *Nicotiana benthamiana* plants. Plants inoculated with wild-type CGMMV-JN was used as a positive control and those buffer-inoculated plants were used as negative control. Plants were grown in greenhouse at 22 °C and 75% humidity under a 16-h photoperiod. Symptoms were checked at 10 days after agroinfiltration, and the virus accumulation levels in systemic leaves were measured by plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) with specific antibody against CGMMV CP [19]. Six plants were agroinfiltrated for each treatment and the experiments were repeated three times.

Site-directed mutagenesis and screening of attenuated strains

The amino acid sequence of CGMMV-JN was aligned with 211 isolates of 23 viruses belonging to *Tobamovirus*. Five amino acids (89 and 114 of CP; 68, 869, and 1069 of RdRp) were found to be completely conserved in the CP and RdRp of tobamoviruses. Amino acids Asp^{89} and Arg^{114} of CGMMV CP were changed to alanine, producing plasmids pCGMMV-CP-D89A (primers: CP89D-A-F/R) and pCGMMV-CP-R114A (CP114R-A-F/R) via site-directed mutagenesis. Meanwhile, Glu^{68}, Lys^{869}, and

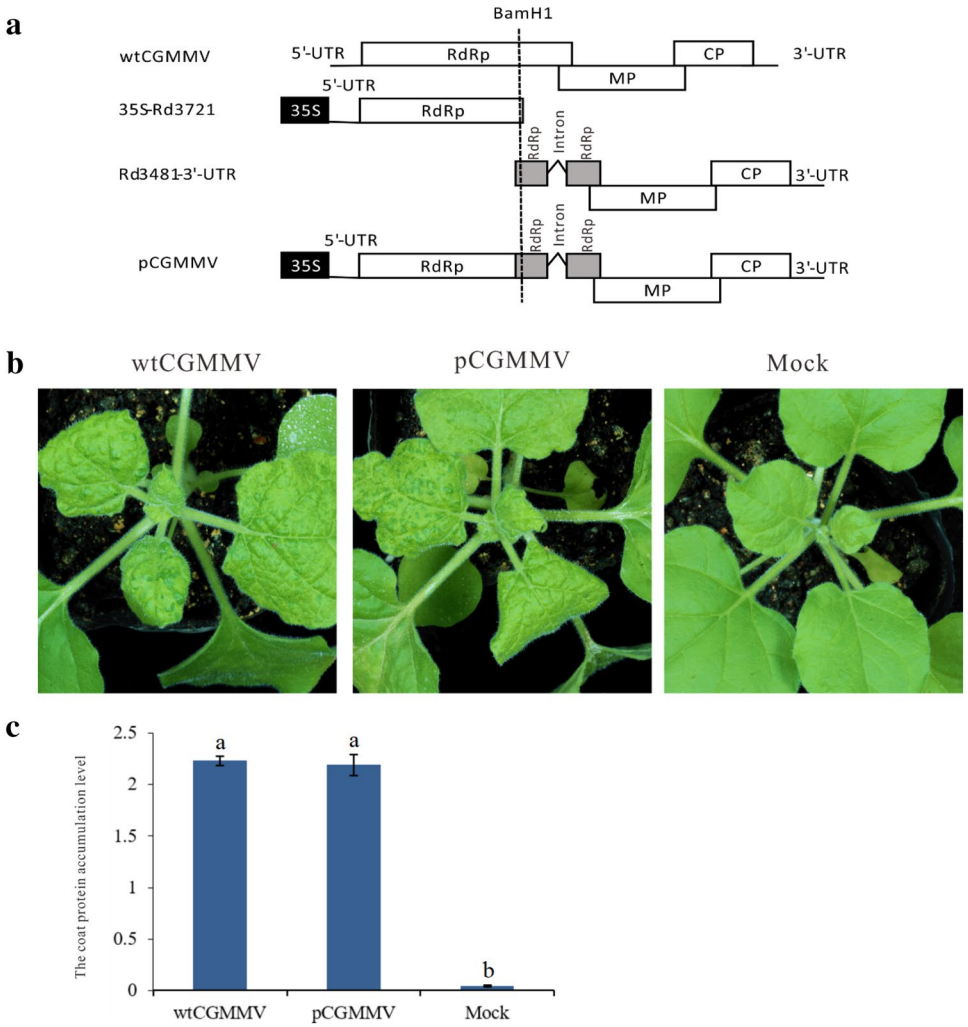

Fig. 1 Construction and infectivity of the clone pCGMMV. **a** Strategy for constructing the clone pCGMMV. wtCGMMV: schematic presentation of the genome organization of TVBMV-JN. 35S-Rd3721 and Rd3481-3′-UTR represented the two intermediate fragments used to construct the full-length cDNA clone pCGMMV. pCGMMV shows the structure of the infectious clone of CGMMV and the position of an intron inserted to stabilize the clone. The mature viral proteins: *RdRp* RNA-dependent RNA polymerase, *MP* movement protein, *CP* coat protein. The 5′-and 3′-untranslated regions (UTR) are depicted. **b** Symptoms of *N. benthamiana* plants inoculated with wtCGMMV and pCGMMV at 10 days post-inoculation. **c** Virus accumulation in the systemically infected leaves as determined by PTA-ELISA at 10 days post-inoculation. Different letters indicate significant differences at $P < 0.05$ and the error bars indicate standard error

Glu^{1069} of CGMMV RdRp were substituted with alanine, producing plasmids pCGMMV-RdRp-E68A (Rd68E-A-F/R), pCGMMV-RdRp-K869A (Rd869K-A-F/R), and pCGMMV-RdRp-E1069A (Rd1069E-A-F/R) (Table 1). These plasmids were transformed into *A. tumefaciens* GV3101, and agroinfiltrated leaves of *N. Benthamiana* plants. Leaves that were mechanically inoculated with parental virus CGMMV-JN was used as the positive control, while those inoculated with buffer served as the negative control. The total RNA was extracted from the upper systemic leaves at 10 days post-agroinfiltration [20] and reverse-transcribed into cDNA by primer CGMMV-SR (5′-TGGGCCCCTACCCCGGGGAAA-3′) and *Moloney Murine leukemia virus* reverse transcriptase (Promega). The accumulation level of viral RNA in the leaves was measured by quantitative Real-Time PCR (qRT-PCR). PTA-ELISA was used to determine the accumulation level of viral protein in the upper systemic leaves (Table 1). Six plants were agroinfiltrated for each treatment and the experiment were repeated four times.

Cross protection assay

The protection effects of attenuated mutants pCGMMV-CP-D89A and pCGMMV-RdRp-E1069A against wild-type CGMMV were evaluated. Both mutants were transformed into *A. tumefaciens* GV3101 and agroinfiltrated *N. benthamiana* leaves. The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying empty vector pCambia0390 were used as a negative control. The systemic leaves were agroinfiltrated with the parental CGMMV-JN at 10 days post-inoculation. Symptoms on the leaves of challenged plants were observed at 10 days after the wild-type CGMMV infection. The accumulation levels of viral RNA were determined by qRT-PCR. The combined extract from all tested plants in each treatment were used for Western blot assay to determine the accumulation levels of viral protein [21]. Six plants were inoculated for each treatment and the experiments were repeated three times.

Table 1 Primers used in the study

Fragment	Primer name	Primer sequence (5′–3′)
(i) Overlapping PCR (pCGMMV construction)		
Fragment 35S-Rd3721	35S-HindIII-F	ACCCAAGCTTCTCGCATGCCTGCAG
	35S-CGMMV-R	AATTAAAACCTCCTCTCCAAATGAAATG
	35S-CGMMV-F	GGAGAGGAGGTTTTAATTTTTATAATTAAAC
	CGMMV-3721-R	TTCTGGGCTTCATTCTT
Fragment Rd3481-3′-UTR	CGMMV-3481-F	GTGCCTACCAAATAGCAA
	CGMMV-IN-3821-R	TCTCACCCTCACCTCTTTATCATAGCTACAAGA
	IN-F	GATAAAGAGGTGAGGGTGAGAAATTGTAAAT
	IN-R	CATATTCCTAAAAGAGAAATGGCTTAC
	CGMMV-IN-3823-F	CATTTCTCTTTTAGGAATATGAATACTCCTGATTTAG
	CGMMV-R	TGCACTCGAGTGGGCCCCTACC
(ii) Mutagenesis and quantitative RT-PCR		
	CP89D-A-F	ACGGCTACGCGTAATAGGGTCATTGAGGTTGTAGA
	CP89D-A-R	ATTACGCGTAGCCGTGGAGCTGAGAAGCGAAA
	CP114R-A-F	CTGTGAAGGCTACTGATGACGCGTCTACAGCC
	CP114R-A-R	TCAGTAGCCTTCACAGCGTTAAGCGACTCAGCAG
	Rd68E-A-F	TCCGGCGTTTTCGATTAGCTTTACCGCCACC
	Rd68E-A-R	CGAAAACGCCGGATACGCATCAGTTACAAGCCT
	Rd869K-A-F	GGAGCGACCGCCGAGATTATAGCGAGGGTCAAT
	Rd869K-A-R	GGCGGTCGCTCCACAACCCGGCACTCC
	Rd1069E-A-F	GTGCATGCAATTCAAGGAGAAACCTTTGAGGAGACG
	Rd1069E-A-R	CTTGAATTGCATGCACAGTATTCACATCATTGTACCCAC
	CGMMV-CP-F	CAGACTCAAGCGGGAAGA
	CGMMV-CP-R	AAGCCCTATCGTAAACATCA
	ef1α-F	TGGTGTCCTCAAGCCTGGTAT
	ef1α-R	ACGCTTGAGATCCTTAACCGC

Statistical analyses

SPSS Statistics for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA) software was used to analyze the data from PTA-ELISA and qRT-PCR (significant difference $P < 0.05$) and calculate confidence interval (CI) level for proportion of the plant developing symptoms in cross protection assay.

Results

Infectivity of intron-containing plasmid pCGMMV

The intron-containing full-length genome of CGMMV-JN was cloned into pCambia0390 to produce the plasmid pCGMMV (Fig. 1a), which was transformed into *A. tumefaciens* and agroinfiltrated into *N. benthamiana* leaves. At 10 days post-agroinfiltration, all the systemic leaves of *N. benthamiana* showed obvious green mosaic mottling symptoms, which were consistent with those of wild-type CGMMV-JN (Fig. 1b). The PTA-ELISA results indicated that systemic leaves of *N. benthamiana* plants inoculated with pCGMMV accumulated same level of CP as *N. benthamiana* plants inoculated with wild-type CGMMV-JN (Fig. 1c).

Symptoms and accumulation levels of five CGMMV mutants

By using site-directed mutagenesis, we introduced mutations to the CP and RdRp encoding region of pCGMMV, producing plasmids pCGMMV-CP-D89A, pCGMMV-CP-R114A, pCGMMV-RdRp-E68A, pCGMMV-RdRp-K869A, and pCGMMV-RdRp-E1069A. After being transformed into *A. tumefaciens* GV3101 and agroinfiltrated leaves of *N. benthamiana*, these plasmids would produce mutants CGMMV-CP-D89A, CGMMV-CP-R114A, CGMMV-RdRp-E68A, CGMMV-RdRp-K869A, and CGMMV-RdRp-E1069A. Like wild-type CGMMV-JN, mutants CGMMV-CP-R114A, CGMMV-RdRp-E68A, and CGMMV-RdRp-K869A produced distinct green mosaic mottling symptoms at 10 days post-agroinfiltration. However, all plants inoculated with mutants CGMMV-CP-D89A and CGMMV-RdRp-E1069A failed to produce any significant symptom (Fig. 2a). The accumulation levels of

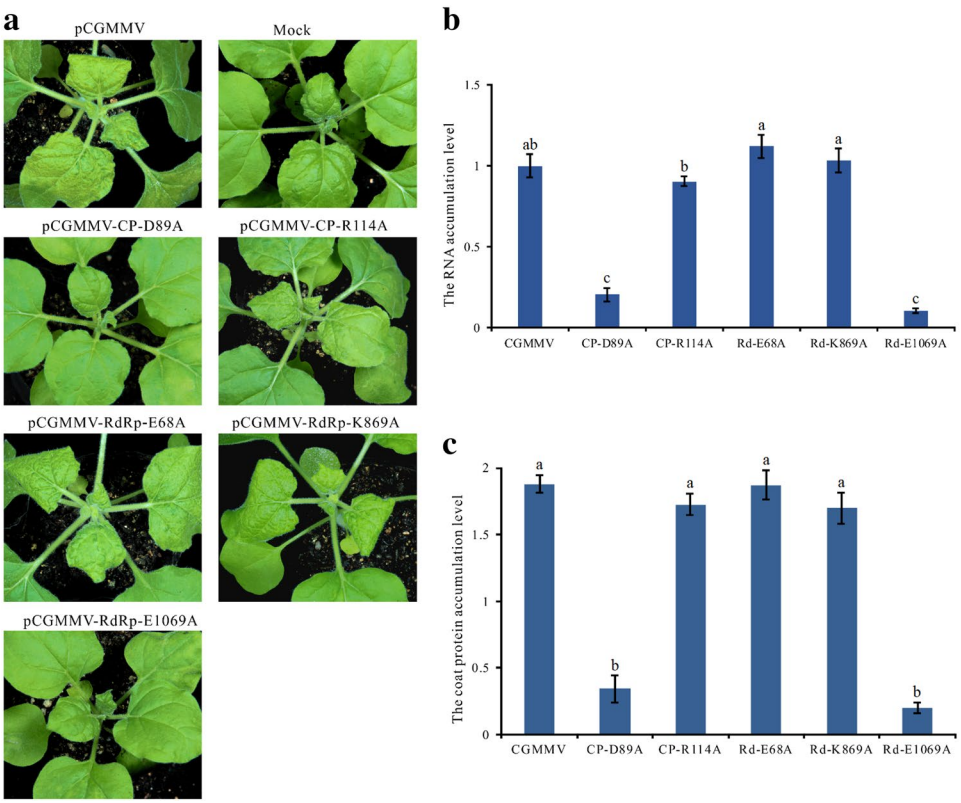

Fig. 2 Symptoms, RNA and CP accumulation levels in *N. benthamiana* plants inoculated with pCGMMV and five mutants. **a** Symptoms in the systemically infected leaves of *N. benthamiana* inoculated with pCGMMV and five mutants. Photographs were taken at 10 days post-inoculation. **b** Detection of the RNA of pCGMMV and the five mutants in the systemically infected leaves by quantitative RT-PCR at 10 days post-inoculation. Levels of *ef1α* transcripts in these tissues were determined as an internal control. Different letters indicate significant differences at $P < 0.05$ and the error bars indicate standard error. **c** The CP accumulation levels in *N. benthamiana* at 10 days post-inoculation, as determined by PTA-ELISA. Different letters indicate significant differences at $P < 0.05$ and the error bars indicate standard error

genomic RNAs of mutants CGMMV-CP-R114A, CGMMV-RdRp-E68A, and CGMMV-RdRp-K869A in the systemic leaves of *N. benthamiana* plants were almost consistent with wild-type CGMMV-JN, while those of mutant CGMMV-CP-D89A and CGMMV-RdRp-E1069A were significantly lower than that of CGMMV-JN ($P < 0.05$) (Fig. 2b). Meanwhile, there was no significant difference in RNA replication levels between mutants CP-D89A and RdRP-E1069A (Fig. 2b). The CP accumulation levels of CGMMV-CP-D89A and CGMMV-RdRp-E1069A were significantly lower than those of CGMMV-JN and mutants CGMMV-CP-R114A, CGMMV-RdRp-E68A, and CGMMV-RdRp-K869A ($P < 0.05$) (Fig. 2c). These results indicated that Asp at position 89 of CP and Glu at position 1069 of RdRp had significant effects on the virulence of CGMMV.

Cross protection assay

As indicated above, the *N. benthamiana* plants inoculated with CGMMV-JN developed green mosaic mottling symptoms at 10 days of post-infection. However, when the plants were pre-inoculated with CGMMV-CP-D89A and CGMMV-RdRp-E1069A 10 days earlier there were no symptoms on them (Fig. 3a). We calculated the results of three repeat experiments and found that only 2/24 plants protected by mutant CGMMV-CP-D89A showed symptoms (95% CI is 0–0.1938) and 0/24 plants protected by mutant CGMMV-RdRp-E1069A showed symptoms, the incidence dropped to 8.3% and 0, respectively. It indicates that when the intervals between induction inoculation and challenging inoculation were 10 days, CGMMV-CP-D89A and CGMMV-RdRp-E1069A could protect *N. benthamiana* plants against severe infection with efficiencies of 91.7% and 100%, respectively. The results of qRT-PCR showed that the accumulation levels of viral RNA in the systemic leaves of protected *N. benthamiana* plants were only 10% of the plants inoculated with CGMMV-JN (Fig. 3b). The Western blot results showed that CP accumulation levels in the systemic leaves of protected *N. benthamiana* plants decreased to a level that undetectable (Fig. 3c). We have amplified the cDNA fragments of CGMMV which including the mutated region and sequencing it at 10 days after challenging inoculation. The results showed that no wild-type virus accumulated in the plants that did not show any symptoms after cross protection and the mutants remained at a low level (22/24 plants protected by mutant CGMMV-CP-D89A and 24/24 plants protected by mutant CGMMV-RdRP-E1069A). However, in the plants showing symptoms of CGMMV, the wild-type virus maintained at a high level and no mutant was detected (Only 2/24 plants protected by mutant CGMMV-CP-D89A) (data not shown).

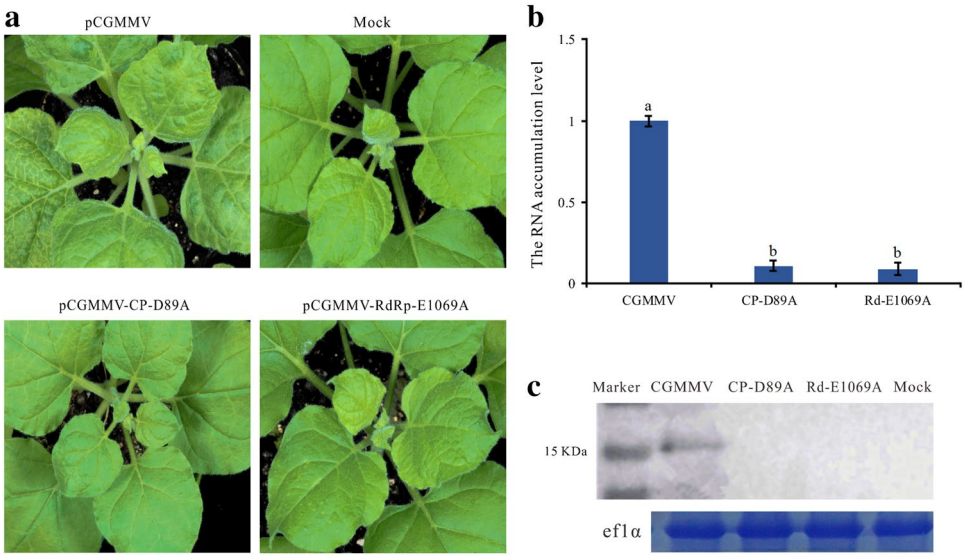

Fig. 3 Symptoms, RNA and CP accumulation levels in *N. benthamiana* plants pre-inoculated with two mutants of pCGMMV-CP-D89A and pCGMMV-RdRp-E1069A in cross protection assay. **a** Cross protection effects of the two mutants against severe infection of wtCGMMV in *N. benthamiana* plants at 10 days post-inoculation. pCGMMV: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying empty vector pCambia0390 and challenged with wild-type CGMMV; Mock: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying empty vector pCambia0390 but not challenged; pCGMMV-CP-D89A: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying mutant CGMMV-CP-D89A and challenged with wild-type CGMMV; pCGMMV-RdRp-E1069A: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying mutant CGMMV-RdRp-E1069A and challenged with wild-type CGMMV. **b** The RNA accumulation level of CGMMV in the systemically infected leaves as determined by quantitative RT-PCR at 10 days post-inoculation in cross protection assay. CGMMV: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying empty vector pCambia0390 and challenged with wild-type CGMMV; CP-D89A: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying mutant CGMMV-CP-D89A and challenged with wild-type CGMMV; Rd-E1069A: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying mutant CGMMV-RdRp-E1069A and challenged with wild-type CGMMV. Levels of *ef1α* transcripts in these tissues were taken as an internal control. Different letters indicate significant differences at $P < 0.05$ and the error bars indicate standard error. **c** The CP accumulation level in *N. benthamiana* at 10 days post-inoculation as determined by Western blot. CGMMV: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying empty vector pCambia0390 and challenged with wild-type CGMMV; Mock: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying empty vector pCambia0390 but not challenged; CP-D89A: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying mutant CGMMV-CP-D89A and challenged with wild-type CGMMV; Rd-E1069A: The *N. benthamiana* plants infiltrated with *A. tumefaciens* GV3101 cells carrying mutant CGMMV-RdRp-E1069A and challenged with wild-type CGMMV

Discussions

In this paper, we successfully constructed an infectious clone of CGMMV, mapped two novel virulence determinants and showed that two attenuated mutants were of great potential on control of severe infection via cross protection.

Infectious cDNA clone is a powerful tool for reverse genetics study. Ooi et al. [22] and Park et al. [23] constructed infectious clone separately by cloning the CGMMV genome under a T7 promoter, which need in vitro transcription before inoculation. Zheng et al. [24] inserted the CGMMV genome between the 35S promoter and ribozyme in the binary plasmid pCN301-CH and developed GFP-tagged cDNA clone of CGMMV. Our clone pCGMMV is also transcriptionally controlled by 35S promoter of CaMV and can be inoculated via agroinfiltration, which is an economic and convenient method [25, 26]. However, the genome of CGMMV-JN was unstable during the cloning and ligation process, due to the recombination and deletion in *E. coli*. It was reported that the promoter-like elements in the viral genome may be recognized by *E. coli* and lead to gene recombination or deletion [25, 27]. This problem could be effectively solved by inserting introns into promoter sequences to ensure the replication stability of the infectious clone [28–30]. To ensure the stability of our plasmid in *E. coli*, an intron of watermelon was inserted after the nucleotide 3821 of CGMMV-JN genome. Such strategy has been adopted frequently in the construction of infectious cDNA clone for potyviruses and *Citrus tristeza virus* [25, 28, 30–32].

There are several reports on the mechanism regulating the mechanism of tobamovirus. Nonsense mutations of replicase and movement protein genes were claimed to contribute to the attenuation of *Tomato mosaic virus* [33]. An attenuated strain of *Tomato mosaic virus* (ToMV),

L11A, has been used in Japan to protect tomato plants against severe infection. By comparing the genomic sequences of L11A and wild virulent strain L, Nishiguchi et al. [34] postulated that three mutations in the 130 kD replicase, i.e., Cys^{348}, Asn^{759}, and Gly^{894} to Tyr, Asp, and Arg, might be responsible for the attenuation of L11. Ichiki et al. [35] showed that changes at amino acid residues Val^{556} (to Thr) and Ser^{760} (to Leu) in 126-kDa protein were responsible for attenuation of *Pepper mild mottle mosaic virus* (PMMoV). Integration of these mutations to parental strain Pa18 of PMMoV produced an enhanced attenuated strain Tpa18ch. All the pepper plants pre-inoculated with Tpa18ch did not show any symptoms 6 weeks after challenge inoculation to leaves and roots [35]. The amino acids responsible for the attenuation of CGMMV-SH33b were postulated to be four amino acids in the replicase of CGMMV [36]. However, the amino acids responsible for the attenuation of VIROG-43 M remains to be determined [15]. In this study, we introduced site-directed mutagenesis to the conserved amino acids of tobamoviruses and mapped two novel virulence determinants of CGMMV aspartic acid at 89 of CP (D_{89}) and glutamic acid at 1069 of replicase (E_{1069}). Mutation of these two amino acids decreased the symptoms severity, accumulation levels of CP and RNA greatly (Fig. 2). We hypothesized that mutations of D_{89} and E_{1069} to alanine might have affected the interaction between CP and replicase with their host or viral partners.

The two mild mutants reported here conferred effective protection against severe infection of CGMMV (Fig. 3). Cross protection can be divided into three stages: the initial stage, the resistance stage, and maintenance stage [37]. In the initial stage, the replication and accumulation of the attenuated strain are relatively low, and generally ineffective at inducing a cross-protective effect on plants. This effect will be induced only after the accumulation of the attenuated strain has reached a certain level. Therefore, an interval period was necessary between the pre-inoculation and challenge infection [4]. In this research, an interval period of 10 days was sufficient for the two mild viruses derived from pCGMMV-CP-D89A and pCGMMV-RdRp-E1069A to elicit cross protection against severe infection.

With the infectious clone of CGMMV constructed in this research, we can screen more attenuated mutants and explore their efficiency of protection against severe infection. These amino acids played important role in virulence of CGMMV. Therefore, the infectious clone of CGMMV and the attenuated mutants obtained here will be of great help to the control of CGMMV via cross protection in the future.

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Author contributions JL and SX designed and performed the experiments, analyzed the data, and wrote the manuscript. X-DL conceived the study and participated in design and coordination and writing. Reagents/materials/analysis tools were contributed by JL, SX, and X-DL. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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